Unexpected Effects of a Heterozygous Dnmt1 Null Mutation on Age-Dependent DNA Hypomethylation and Autoimmunity

Raymond Yung,[1] Donna Ray,[1] Julie K. Eisenbraun,[1] Chun Deng,[1] John Attwood,[1]
Michael D. Eisenbraun,[2] Kent Johnson,[3] Richard A. Miller,[3] Samir Hanash,[4] and Bruce Richardson[1,5]

Departments of [1]Internal Medicine, [2]Cell and Molecular Biology, [3]Pathology, and [4]Pediatrics and Communicable Diseases,
University of Michigan, Ann Arbor.
[5]Ann Arbor Veterans Affairs Hospital, Michigan.

DNA methylation modifies gene expression. Methylation patterns are established during ontogeny, but they change with aging, usually with a net decrease in methylation. The significance of this change in T cells is unknown, but it could contribute to autoimmunity, senescence, or both. We examined the effects of a null mutation in DNA methyltransferase 1 (Dnmt1), a gene maintaining DNA methylation patterns, on immune aging. Whereas aged control mice developed hypomethylated DNA, autoimmunity, and signs of immune senescence as predicted, the knockout mice surprisingly increased DNA methylation and developed signs of autoimmunity and senescence more slowly. To identify potential mechanisms, we compared transcripts of DNA methyltransferase and methylcytosine binding protein family members in control and knockout mice. MeCP2, a methylcytosine binding protein involved in gene suppression and chromatin inactivation, was the only transcript differentially expressed between old knockout mice and controls, and thus it is a candidate for a gene product mediating these effects.

D NA methylation modifies gene expression by targeting methylcytosine binding proteins to specific promoter sequences, which suppresses gene expression directly, or indirectly by promoting chromatin inactivation through transcriptional repression domains (1). DNA methylation patterns are established during development in a tissue-specific fashion (2), and they serve to suppress the expression of genes not essential or potentially detrimental to cellular function (3,4). Our group has examined the role DNA methylation plays in regulating T-lymphocyte function and gene expression. We reported that inhibiting T-cell DNA methylation with DNA methyltransferase (DNA MTase) inhibitors such as 5-azacytidine modifies gene expression and induces autoreactivity, and that T cells made autoreactive by this mechanism induce an autoimmune disease in vivo. Abnormalities resulting from the autoreactive cells include anti-DNA antibodies, immune complex renal disease, lymphocytic interstitial pneumonitis, and an autoimmune liver disease resembling primary biliary cirrhosis (5,6). These observations suggest that T-cell DNA hypomethylation can contribute to autoimmunity. In other studies, our group and others have reported that T-cell DNA demethylates with age (7,8), similar to many but not all tissues (9). Significantly, aging mice also develop anti-DNA antibodies and T-cell-dependent autoimmune lesions in several organs, including the lung and liver (10,11), which resemble those induced by experimentally demethylated T cells. This raises the possibility that age-dependent changes in T-cell DNA methyla-

tion may also contribute to the development of autoimmunity in aged mice.

T-lymphocyte function also declines with age. These changes include decreased interleukin-2 (IL-2) production and the accumulation of "memory" T cells (12). Because DNA methylation patterns change with aging, altered DNA methylation is an attractive explanation for age-dependent changes in cellular function. However, despite evidence for age-dependent changes in DNA methylation, there is little information available regarding the functional significance of the changes, or their relevance to immune senescence.

The inactivation of methylation genes in mouse models permits a correlative assessment of questions regarding the relationship between DNA hypomethylation and autoimmunity, as well as the functional significance of age-dependent changes in DNA methylation. A heterozygous DNA methyltransferase 1 (Dnmt1) "knockout" mouse has been described (13). Dnmt1 is an enzyme responsible for maintaining DNA methylation patterns through mitosis (14). Homozygosity for the null allele is lethal to the embryo, whereas the heterozygous knockout mouse is phenotypically normal but has hypomethylated DNA (13). We hypothesized that the loss of one Dnmt1 allele might increase the rate of the age-dependent decrease in T-cell deoxymethylcytosine (d^mC) content, resulting in an increase in the rate of onset of age-dependent autoimmunity. We also hypothesized that age-dependent changes in T-cell function and gene expression would be increased in the Dnmt1-deficient mice, as a result

of an effect of the Dnmt1 mutation on methylation changes with aging.

To test these hypotheses, we compared the development of histologic and serologic signs of autoimmunity in young, middle aged, and old heterozygous Dnmt1 knockout mice with their wild-type littermates. Selected parameters of T-cell function and gene expression known to change with aging were also compared. Additional studies included a comparison of total genomic d^mC content and expression of DNA MTase and methylcytosine binding protein transcripts between groups. The results support the association between age-dependent, progressive DNA hypomethylation and the development of autoimmunity. Surprisingly, however, the Dnmt1 knockout mice increased genomic d^mC content with aging, and they showed a decline in the development of several age-dependent immune abnormalities. A search for possible secondary effects of the knockout mutation showed that these mice maintained MeCP2 expression with aging, which may maintain patterns of DNA methylation and gene expression with aging. (MeCP2 is a methylcytosine binding protein involved in gene suppression and chromatin inactivation.)

MATERIALS AND METHODS

Mice

Heterozygous female Dnmt1 knockout mice, bred onto a C57BL/6 background, and their wild-type littermates were obtained from Jackson Labs (Bar Harbor, ME) and housed in a specific pathogen-free environment in the University of Michigan Unit for Laboratory Animal Medicine. Sentinel mice from this colony were tested every 3 months for antibodies to murine viral pathogens, and all tests were negative during the experimental period. At the time of the study, the groups were aged approximately 6, 11, and 18 months, with a variability of ±1 month.

Histologic Analysis

Tissues were fixed, embedded in paraffin, sectioned, and stained with hematoxylin and eosin, using previously published protocols (5,6). Slides were read by a pathologist who was blinded to the groups being studied, and they were scored on a 0–4+ scale. Activity of glomerulonephritis was scored according to published criteria (15).

Isolation of Lymphocytes and Measurement of Lymphocyte Function

Spleens were removed from mice and splenocytes recovered as described (5). Where indicated, CD4+ T cells were enriched by two cycles of treatment with anti-CD8 plus anti-Ia^b (Pharmingen, San Diego, CA) and rabbit complement (Pel-Freeze, Brown Deer, WI) and previously published protocols (5). This typically resulted in a twofold to threefold increase in the percentage of CD4+ cells. Proliferative and IL-2 responses were measured by using flat-bottomed microtiter wells (Costar, from Corning; Corning, NY) and protocols described by others (16). Briefly, 50 μl of anti-CD3 (10 μg/ml in phosphate-buffered saline; 2C11, from Pharmingen) were added to the microtiter wells, and allowed to bind overnight at 4°C. The wells were then washed three to four times with phosphate-buffered saline (PBS), and 10^5 cells were added to each well in 200 μl of Roswell Park Memorial Institute 1640 (RPMI) supplemented with 10% fetal calf serum (FCS) and 50 μM of 2-mercaptoethanol. Where indicated, anti-CD28 (Pharmingen, 2.25 μg/ml) was also added; 150 μl of media was removed at 48 hours, saved for enzyme-linked immunosorbent assays (ELISAs), and replaced with an equal volume of fresh media. At 54 hours, 1 μCi of tritiated thymidine (^{3}H-TdR) was added, and the cells were harvested 2 hours later and proliferation measured by ^{3}H-TdR incorporation. IL-2, IL-4, IL-10 and interferon gamma (IFN-γ) in culture supernatants were detected by two-site ELISA, using matched pair antibodies (Pharmingen) according to the manufacturer's instructions. Levels were determined graphically by using a standard curve generated with recombinant murine cytokines (Genzyme, Cambridge, MA).

Flow Cytometric Analysis

Phenotypic characterization was performed by using anti-CD4-fluorescein isothiocyanate (FITC), anti CD8-FITC, anti-CD11a-FITC, and anti-CD44-phycoerythrin (PE) (all from Pharmingen) as described by Quddus and colleagues (5). Controls included MSIg-PE and mouse serum immunoglobulin (MSIg)-FITC. All samples were analyzed by using a Coulter ELITE flow cytometer.

Autoantibodies

Sera were diluted 1:200 with PBS, and then they were added to Immulon 4 flat-bottomed microtiter wells (Dynex Technologies, VA) coated with ssDNA or dsDNA as described (5,6). The plates were then washed and bound immunoglobulin (Ig) detected with horse radish peroxidase (HPO)-conjugated polyvalent goat antimouse IgM or IgG (Sigma, St. Louis, MO) as described (5,6). All determinations were performed in quadruplicate and repeated at least once. A reference standard consisting of pooled sera from mice with lupus (17) was included on each plate, and results are expressed relative to this standard. Total IgG and IgM were also measured by ELISA, using previously published protocols (5,6).

Quantitation of Genomic Deoxymethylcytosine Content

The d^mC content was compared between groups by using *Sss*I methylase assays (18). Briefly, 500 ng of genomic DNA from CD4-enriched T cells was incubated 37°C for 4 hours with 4 U of *Sss*1 CpG methylase (New England BioLabs, Beverly, MA), 1.5 μM of *S*-adenosyl-*L*-[methy-^{3}H] methionine (^{3}H-SAM) (77 Ci/mmol; Amersham, Arlington Heights, IL), and 1.5 μM of nonradioactive *S*-adenosylmethionine (New England BioLabs) in buffer provided by the manufacturer. Five μl of 2.5-mM nonradioactive SAM was then added and the reaction mixture was spotted on Whatman GF/C 2.4-cm^2 filter disks, which were air dried for 15 minutes. The filters were washed with 6 ml of 5% TCA followed by 6 ml of 70% ethanol, and they were air dried overnight; ^{3}H incorporation was measured by using a scintillation spectrometer. All determinations were performed in triplicate, and results were normalized to the young control

group, arbitrarily defined as 100%. Similar studies were performed on brain tissue from old mice.

RT-PCR Amplification of DNA Methyltransferase and Methylcytosine Binding Protein Families

Tissue stored in liquid nitrogen was rapidly thawed, placed in Trizol (GIBCO-BRL, Gaithersburg, MD) and homogenized. Total RNA was purified according to the manufacturer's protocol. Real-time reverse transcription polymerase chain reaction (RT-PCR) was performed with a LightCycler (Roche, Indianapolis, IN). With the use of a One-Step RNA Amplification Kit with SYBR Green I (Roche), 200 ng of total RNA was converted to cDNA and then amplified in one step. Conditions for the reactions were as follows: reverse transcription at 55°C for 10 minutes; denaturation at 95°C for 30 seconds; amplification at 95°C for 1 second, 56°C for 10 seconds, and 72°C for a period determined by the product length (1 second per 25 nucleotides) for a total of 45 cycles. At the completion of amplification, melting characteristics of the product were determined. In each experiment, water was included as a negative control to rule out primer dimer formation. A series of five dilutions of one RNA sample were also included to generate a standard curve, and this was used to obtain relative concentrations of the transcript of interest in each of the RNA samples. A standard curve was generated during each experiment by using one of the primer pairs. Relative concentrations were then determined by the second derivative method with the LightCycler computer software. Amplification of GAPDH was performed to confirm that equal amounts of total RNA were added for each sample and that the RNA was intact and equally amplifiable among all samples.

Primer sequences were as follows, listed as forward; reverse:

GAPDH: 5′-TTG AAG GGT GGA GCC AAA CG; 5′-TGG GAG TTG CTG TTG AAG TCG

Dnmt1: 5′-AGG ATG AGA GGG AGG AGA AGA GAC; 5′-GGT GGA GTC GTA GAT GGA CAG TTT C

Dnmt3a: 5′-CAC CAG CCA AGA AAC CCA GAA AG; 5′-TCC CAT CAA AGA GAG ACA GCA CG

Dnmt3b: 5′-CAA ACC CAA CAA GAA GCA ACG AG; 5′-CCA GAC ACT CCA CAC AGA AGC ATC

Mbd1: 5′-CGT TTG GAC GCT CAG ACA TC; 5′-TCC TCT TTC ACC AGG CAA GC

Mbd2: 5′-CAC GAA CCA CCC GAG CAA TAA G; 5′-TCA GTG CCT CCT CCA GTT TCT TG

Mbd3: 5′-ACC CCA GCA ACA AGG TCA AG; 5′-TTT GGA AAG GAG GTG GAC G

Mbd4: 5′-ACT CGG CTT GCT TGC TAT TG; 5′-TGA AGG ATT TGC GTC TTG GG

MeCP2: 5′-CAA CCT TCA GCC CAC CAT TC; 5′-TCT CCA GGA CCC TTT TCA CC.

Statistical Analysis

The difference between means was tested by using the unpaired Student's *t* test with pooled variance, calculated with Systat and Sigmastat software (SPSS Science, Chicago, IL). Linear regression and analysis of variance was similarly calculated with Systat and Sigmastat software. Differences between grouped assays were tested by using Fisher's Method of Combining Independent Tests (19). Fisher's Exact Test was used to test the significance of histologic data.

RESULTS

Development of Autoimmunity

The effect of the Dnmt1 null mutation on the long-term health of mice is unknown, so weights and overall health were compared among young, middle aged, and old knockout mice and controls. No differences in weights, general health, or mortality were observed among groups during the duration of this study. Complete autopsies were performed on 6 mice from each of the 6 groups (young, middle aged, and old; knockout and control), and no evidence for malignancy was identified. No splenomegaly was identified in any of the groups, and total numbers of splenocytes were similar between the control and old knockout mice ($6.92 \pm 0.48 \times 10^7$ vs $6.54 \pm 0.98 \times 10^7$, mean $\pm$ SEM of six mice/ group, control vs knockout). This suggests that the Dnmt1 mutation has no major effect on the health of the mice at these ages.

The development of autoimmunity in the Dnmt1 knockout mice and their wild-type littermates was compared. At later ages, C57BL/6 mice develop lymphocytic infiltrates of the salivary glands and liver, as well as of other organs (10), so histologic evidence for these lesions was sought in young, middle aged, and old Dnmt1 knockout and control mice. The most consistent abnormalities detected were mononuclear infiltrates of the liver and salivary glands. Figures 1A and 1B show representative histology of these organs from affected mice. By 18 months of age, a majority of mice from both groups had evidence for autoimmune salivary gland lesions. These results compare favorably with those reported by others (10). However, in the 11-month-old age group, significantly ($p = .02$) more of the control mice had moderate to severe lesions compared with the knockout mice (Table 1). These lesions are typically ~86% T cells and 7% B cells in aged C57BL/6 mice (10), although the composition of the lesions was not determined in the Dnmt1 knockout mice. The liver lesions were observed in 5 of 6 old control mice, whereas only 1 of 6 old knockout mice had the mononuclear cell infiltrates. Four of the six aged control mice had evidence for glomerulonephritis and interstitial inflammation (average activity score 3.8 ± 1.5, mean $\pm$ SEM), whereas 2 of the 6 aged knockout mice had similar lesions (activity 1.7 ± 1.1). Three of six of the old control mice had mild to moderate lymphocytic infiltration of the lungs; none of six old knockout mice did. It is noteworthy that in all organs, the controls had greater evidence
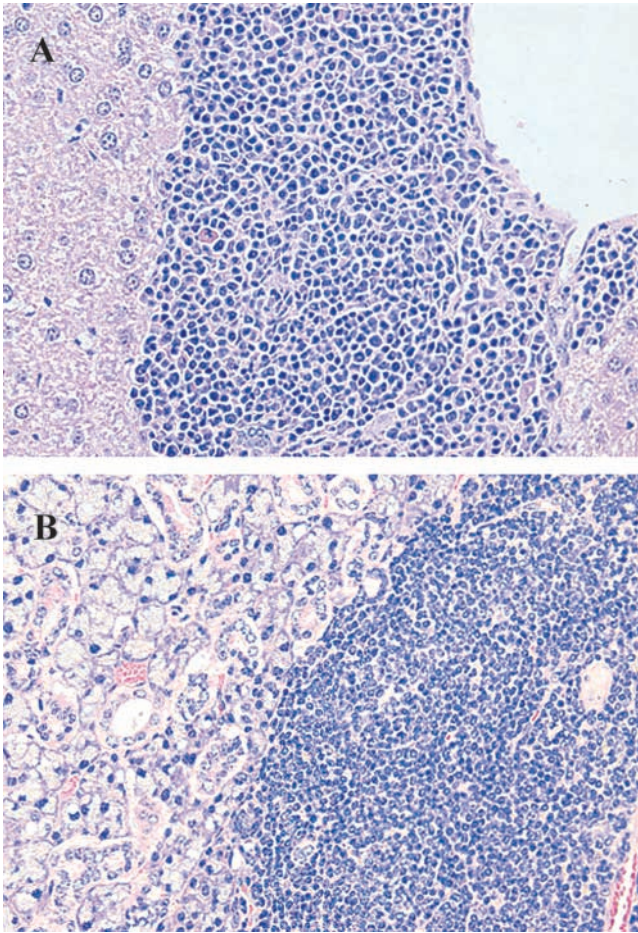
Figure 1. Histologic characterization of autoimmunity. The livers and salivary glands were removed from Dnmt1-deficient or wild-type littermates at approximately 6, 11, and 18 months of age. Tissues were fixed and sectioned, and then stained with hematoxylin and eosin. **A** shows a representative section from the liver, and **B** shows one from the salivary glands of 18-month-old control mice (400×).

knockout and control mice, and they were tested for IgM and IgG anti-ssDNA and anti-dsDNA antibodies (Figure 2). No difference in titer was observed in the young and middle aged mice. However, in all cases, sera from the aged control mice had greater titers of anti-ssDNA ($p < .05$) and anti-dsDNA ($p < .025$) antibodies relative to their Dnmt1-deficient littermates ($p < .005$ overall). Total IgM and IgG were also measured in the aged groups, to exclude the possibility that an increase in autoantibodies reflected a polyclonal increase in immunoglobulin. ELISA assays demonstrated no significant differences in total IgM (OD 0.377 ± 0.007 vs 0.363 ± 0.004, old knockouts vs old controls, mean $\pm$ SEM, using a 1:100 serum dilution) or IgG (OD 0.456 ± 0.005 vs 0.447 ± 0.009). No significant differences in total IgG or IgM levels were observed in the young or middle aged groups as well (not shown). Together, these data indicate that the wild-type mice develop anti-DNA antibodies and tissue evidence for autoimmunity more rapidly than the Dnmt1 knockout mice.

Immune Characterization

The observation that autoimmunity developed more slowly in the Dnmt1-deficient mice raised the possibility that the knockout mice had impaired T-cell function, thus preventing the development of autoimmunity. It was also possible that the Dnmt1 null mutation modified the development of immune senescence. As a way to test these possibilities, T-cell subsets, proliferative responses, and cytokine secretion, all reported to change with aging (12), were com-
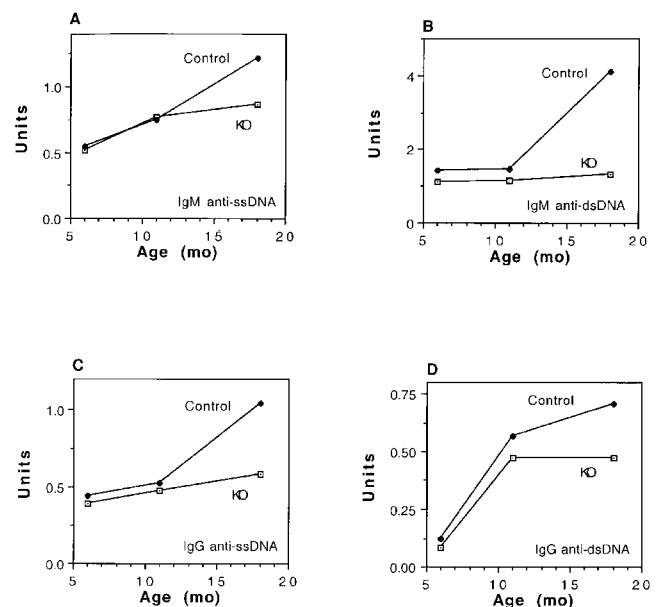
for autoimmunity compared with their knockout littermates ($p < .01$ overall by Fisher's method for combining independent tests). Overall the incidence of these lesions is somewhat higher than those reported previously (10). However, in the present study, tissues were counted as positive if lymphocyte aggregates were observed in the sections, whereas in the previous report (10), tissues were only scored as positive if more than 50 mononuclear cells were observed in the center of the tissue at 100× magnification.

Serologic evidence for autoimmunity was also sought. Sera were obtained from young, middle aged, and old

Figure 2. Anti-DNA antibodies in Dnmt1-deficient and control mice. Sera were obtained from Dnmt1-deficient (knockout, or KO; ▫) or wild-type littermates (control; ◆) at the indicated ages, and they were tested for the following autoantibodies: **A**, IgM anti-ssDNA; **B**, IgM anti-dsDNA; **C**, IgG anti-ssDNA; and **D**, IgG anti-dsDNA. Results represent the mean of quadruplicate determinations performed on 10 mice/group and are expressed relative to a standard consisting of sera from mice with lupus induced with a DNA methylation inhibitor. Standard errors averaged 15.6% of the means.

Table 1. Salivary Gland Inflammation: Middle-Aged Mice

Mouse Strain	Inflammation*		
	Negative	Mild	Moderate–Severe
Control	1	1	4
Knockout	4	3	0

*$p = .02$, negative + mild vs moderate + severe.

pared between groups. Flow cytometric analysis was used to compare the percentage of CD4+ and CD8+ T cells in the spleen in the young, middle aged, and old knockouts and controls. No significant differences were observed at any age (not shown). Others have reported that "memory" CD4+ T cells, expressing high levels of CD44, accumulate with age (12), so CD44 expression was compared between groups (Figure 3). Overall, the number of CD4+ memory cells increased with age in both groups ($p = .001$ by analysis of variance, or ANOVA). However, T cells from the knockout mice tended to have fewer CD4+, CD44 high T cells than controls ($p = .031$ overall by ANOVA), which reached statistical significance in the old group ($p = .003$ by t test), but not at the younger ages, suggesting a more pronounced difference in the older mice.

Proliferative responses were tested by stimulating unfractionated splenocytes and CD4-enriched splenocytes from young and old Dnmt1 knockout mice and controls with immobilized anti-CD3 with and without anti-CD28 (Figure 4). No differences were seen between the young groups with either form of stimulation. The control group showed essentially no change in response to either stimulus with aging. Although murine T-cell proliferative responses usually decline with age by 18 months of age (12), the decline appears to be delayed when immobilized anti-CD3 is used as the stimulus (16), perhaps reflecting a relatively stronger stimulus from immobilized anti-CD3. In contrast, the CD4-enriched population from the knockout mice demonstrated an increased proliferative response with age to anti-CD3 with and without anti-CD28 ($p < .05$, young vs old). These responses were significantly greater than those in the age-matched controls ($p < .005$ overall).

Cytokine responses were also compared by using CD4-enriched populations from the young, middle aged, and old knockouts and controls. The cells were stimulated with immobilized anti-CD3 and soluble anti-CD28 as in Figure 4,
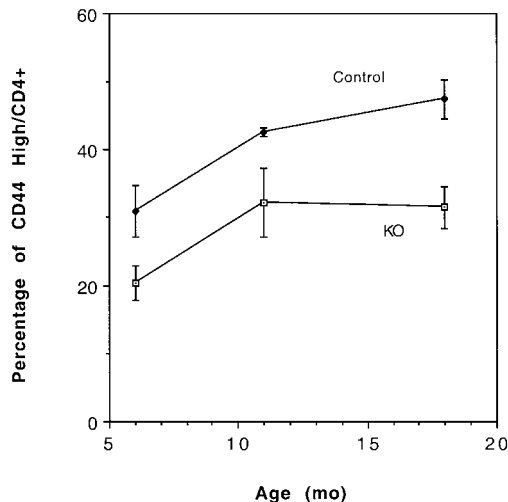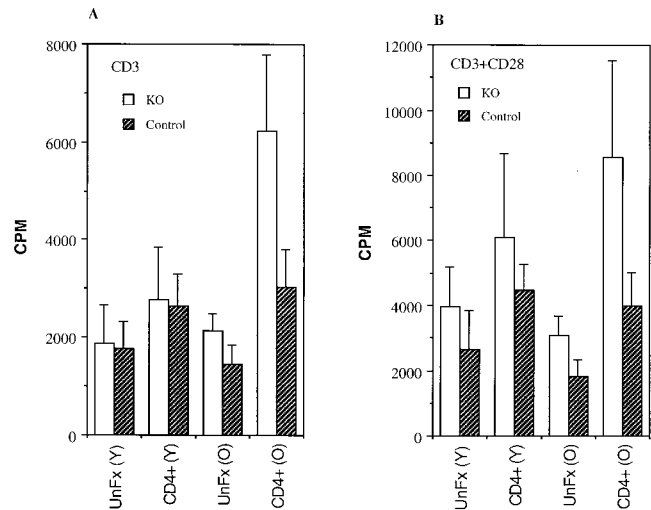

Figure 4. T-cell proliferative responses in Dnmt1-deficient and control mice. **A**, Unfractionated splenocytes (UnFx) or CD4-enriched splenocytes (CD4+) from young (Y) and old (O) knockout (KO, open bars) and control (hatched bars) mice were stimulated with immobilized anti-CD3. **B**, Similar preparations were stimulated with immobilized anti-CD3 and soluble anti-CD28. In both panels proliferation was measured 54 hours later by ^{3}H-TdR incorporation. Results represent the mean $\pm$ SEM of 5–7 young and 5–7 old mice. CPM = counts per minute.

and supernatants harvested 48 hours later. T cells from the control mice showed an age-dependent decline in IL-2 production (Figure 5) as described by others (12; $p < .005$ young vs old), whereas no significant decrease was observed in the knockouts. The difference in the slopes (young to old) was significant (-0.480 ± 0.099 vs $-0.029 \pm$
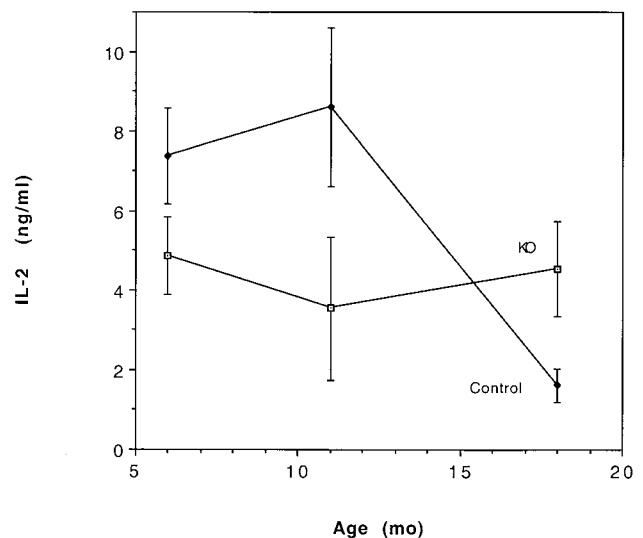

Figure 3. Development of the CD4+ memory subset in Dnmt1-deficient and control mice. Splenocytes from Dnmt1-deficient (knockout, or KO; ▫) and control (control; ◆) mice were stained with anti-CD4-FITC and anti-CD44-PE and then analyzed by flow cytometry. Results represent the mean $\pm$ SEM of 8–12 mice/point.

Figure 5. IL-2 secretion by CD4-enriched T cells from Dnmt1-deficient and control mice. T cells from knockout (KO; ▫) or control (control; ◆) mice were stimulated with anti-CD3 + anti-CD28 as in Figure 3, and 48 hours later supernatants were harvested and tested for IL-2 by enzyme-linked immunosorbent assay. Results represent the mean $\pm$ SEM of 6–7 young, 3–4 middle aged, and 7 old mice per group.

0.129, control vs knockout, $p < .02$). The amount of IL-2 secreted by T cells from control and Dnmt1 knockout mice was also significantly different in the old group ($p = .041$). Although there was a trend for the knockout mice to secrete less IL-2 in the younger age groups, this did not achieve statistical significance. No significant differences were observed in IL-4, IL-10, or IFN-γ production between T cells from both young and old groups (not shown). These results argue against impaired T-cell responsiveness as a mechanism for the protection of autoimmunity developing in the knockout mice. In addition, the observation that the knockout mice develop signs of autoimmunity more slowly, develop fewer "memory" cells, and do not lose IL-2 secretory capacity by 18 months of age suggests that some aspects of the immune system may age more slowly in the knockout mice.

DNA Methylation Analysis

The effect of age on genomic d^{m}C content was compared in the knockout mice and controls. DNA from the CD4-enriched splenocytes was incubated with ^{3}H-SAM and *Sss*I methylase, and then it was precipitated and washed; ^{3}H incorporation was compared between groups. *Sss*I will methylate all unmethylated CG pairs, so ^{3}H incorporation is inversely proportional to d^{m}C content, with greater ^{3}H incorporation reflecting a lower d^{m}C content. Figure 6A shows that lymphocyte DNA from control mice demethylates in an age-dependent fashion as described by others (8,20,21). In addition, DNA from young Dnmt1 knockout mice is relatively hypomethylated compared with that of age-matched littermates ($p < .01$), which is consistent with
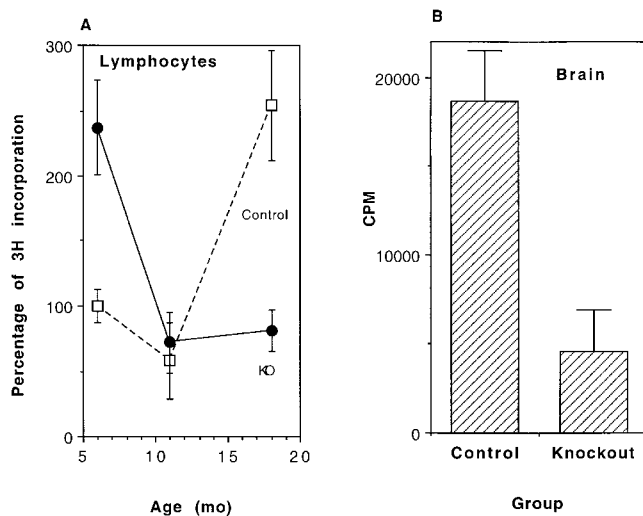

Figure 6. Genomic DNA methylation in CD4-enriched T cells from Dnmt1-deficient and control mice. **A**, DNA from lymphocytes was isolated from knockout mice (—) or controls (- - -), and it was labeled with ^{3}H-SAM, using *Sss*I methylase. The DNA was then precipitated and washed, and ^{3}H was incorporation measured. All determinations were performed in triplicate. The results represent the mean $\pm$ SEM of determinations performed on 4–5 mice/data point, normalized to young control mice. **B**, Similar studies were performed on DNA from brains of old knockout mice and controls. Results represent the mean $\pm$ SEM of [^{3}H]-CH$_3$ incorporation into the DNA from 3 mice/group.

published reports (13,22). Unexpectedly, however, total d^{m}C content increased with age in the knockout mice, so that by 18 months the d^{m}C content was significantly greater ($p < .01$) than in age-matched littermates, and approximated that of the young wild-type mice. Similar results were observed in the brains of the aged mice ($p < .02$, Figure 6B), suggesting the existence of compensatory mechanisms for Dnmt1 deficiency.

Expression of DNA Methyltransferase and Methylcytosine Binding Protein Familes

Because brain tissue from the aged knockout mice demonstrated greater amounts of d^{m}C than did age-matched controls, similar to lymphocytes, levels of DNA MTase and methylcytosine binding protein transcripts were compared by real-time RT-PCR in brains from young and old knockout and control mice, using 3 mice/group. There was a small and statistically insignificant decrease in Dnmt1 mRNA in brains from young knockout mice relative to controls (96 $\pm$ 5 vs 132 $\pm$ 16, mean $\pm$ SEM, knockout vs control), and similarly no significant difference in the brains from old mice (106 $\pm$ 25 vs 112 $\pm$ 27). Similar results were found for Dnmt3b, whereas Dnmt3a mRNA was barely detectable in all groups, requiring $\sim$30 cycles of amplification for detection. Methylcytosine binding proteins have been implicated in modifying chromatin structure and gene expression (1,23), so expression of MBD1–4 and MeCP2 transcripts were compared. No significant differences in MBD1, MBD2, or MBD4 mRNAs were found between groups as well. MBD3 mRNA demonstrated a significant decrease with aging in the control ($p = .03$) and knockout ($p = .008$) groups (Figure 7A). However, because the change was identical in both groups, it is unlikely that this gene product contributes to the phenotypic differences between the controls and knockout mice. In contrast, although MeCP2 decreased with age in the control mice ($p = .001$), no decrease was observed in the knockout mice, and the difference in MeCP2 levels between old control and knockout mice was significant (Figure 7B; $p = .03$). GAPDH was run as a control and showed no significant differences between groups.

DISCUSSION

These experiments were designed to determine the effects of heterozygous Dnmt1 deficiency on lymphocyte DNA methylation with aging, and to correlate the results with the development of autoimmunity and immune senescence. Four significant observations were made. First, whereas lymphocyte DNA from the young Dnmt1 knockout mice was hypomethylated relative to age-matched controls, total d^{m}C content increased with age in the knockouts. This is in contrast to the wild-type littermates, which demonstrated the expected age-dependent decrease. Second, the onset of age-dependent autoimmunity was delayed in the knockout mice compared with controls. Third, characteristics of T-cell aging, in particular development of the memory subset and the rate of decrease in IL-2 secretion, showed altered developmental patterns in the knockout mice. Fourth, levels of MBD3 mRNA decreased with age in both control and knockout mice, whereas MeCP2 mRNA decreased only in the control mice.
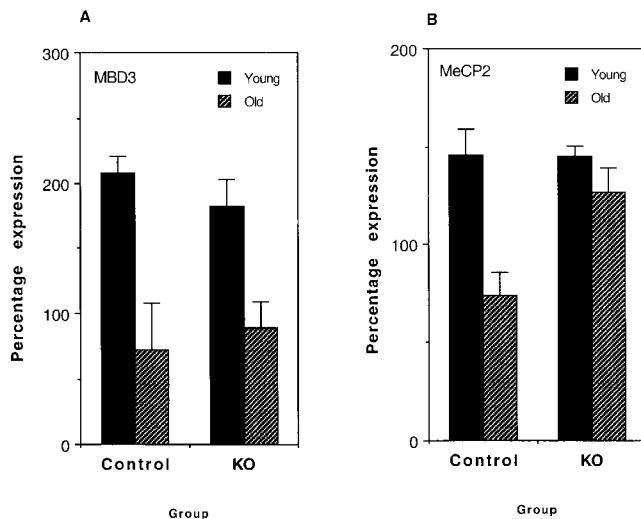
Figure 7. Effect of age on MBD3 and MeCP2 expression in brains from Dnmt1-deficient and control mice. **A**, MBD3 mRNA was quantitated as described in Materials and Methods from brains of young (solid bars) and old (hatched bars) control or knockout mice. Results represent the mean ± SEM of 3 mice/group. **B**, MeCP2 mRNA was quantitated from the same groups as described in **A**.

The relative hypomethylation of lymphocytes from the young knockout mice was not unexpected, because others have reported similar findings (22). However, the observation that levels of Dnmt1 transcripts in the knockouts were identical to controls was surprising. Because Dnmt1 levels are decreased in the Dnmt1 knockout embryonal stem cells (13), it is possible that a decrease in Dnmt1 exists during development, resulting in hypomethylation that is maintained at least through 6 months of age despite higher levels of Dnmt1 transcripts. This is consistent with the observation that the increase in methylation is delayed and not observed until 11 months of age.

The changes in genomic d^{m}C content observed in both groups could potentially affect gene expression. However, phenotypic changes occurred largely in the control group. Therefore, it seems reasonable to propose that the changes in the control group occur in transcriptionally relevant regions of the genome. Other work from our group demonstrates that in mice, age-dependent methylation changes occur outside of CpG islands (manuscript in preparation), suggesting the changes in methylation occur in promoters lacking islands. Slightly less than half of murine genes lack CpG islands (24), so the changes could potentially affect a large number of genes. In contrast, the paucity of functional changes in the knockout group suggest that the increases in d^{m}C may occur largely outside of regulatory regions.

The age-dependent increase in DNA methylation observed in the knockout mice could be viewed as paradoxical, because expression of only one Dnmt1 allele would be expected to lead to a more pronounced decrease in DNA methylation with age. However, the increase in DNA methylation observed in the knockout mice may represent a compensatory mechanism, for which there is precedent. Our group has reported that inhibiting T-cell DNA methylation

with pharmacologic inhibitors such as 5-azacytidine or procainamide results in a compensatory increase in Dnmt1 mRNA expression and increased DNA MTase enzyme activity (18,25). This is supported by our observation that brain Dnmt1 mRNA levels were not significantly different between groups. It is thus possible that similar regulatory mechanisms lead to overexpression of the remaining Dnmt1 allele. Two additional DNA MTases with de novo methylation capabilities, Dnmt3a and Dnmt3b, have recently been described (26), but minimal Dnmt3a mRNA was detectable in the brains of the mice, and no significant differences in Dnmt 3b mRNA were observed. However, as gene expression at the protein level was not measured, it is possible that differential expression in the activity of one or more of these enzymes might be responsible. It is also possible that a relative increase in a demethylase in the control mice could account for progressive hypomethylation in this group. A recent report suggests that methylcytosine binding domain 2 (MBD2) might have demethylase activity, although others have been unable to confirm this result (27–29). It is thus relevant to note that there were no differences in MBD2 transcripts between groups. Others have described a ribonucleoprotein complex with glycosidase activity in chickens, which results in DNA demethylation (30). The differential expression of a similar enzyme in mammals might account for the decrease in d^{m}C content observed in the control mice. Future experiments will have to compare expression of these enzymes at the protein level in various tissues from young, middle aged, and old Dnmt1 knockout mice and controls under both quiescent and mitogen-stimulated conditions, to test these possibilities.

Comparison of transcripts of the methylcytosine binding protein family demonstrated that MBD3 decreased with age in both controls and knockouts, and thus is unlikely to contribute to the differences in age-dependent DNA methylation between the groups. However, MeCP2 transcripts decreased in the controls but not the knockouts. This protein binds methylcytosine and contains a transcriptional repression domain that interacts with a transcriptional corepressor complex containing histone deacetylases (31). MeCP2 can also suppress gene expression through mechanisms independent of histone deacetylases (32). A decrease in this protein could potentially lead to abnormal expression of genes repressed by both mechanisms, contributing to the aging process. It is also possible that persistance of its expression may contribute to an increase in d^{m}C with age in the knockout mice by promoting chromatin inactivation. This hypothesis is indirectly supported by the observation that the knockout mice are phenotypically unchanged with aging despite increasing d^{m}C levels, consistent with progressive methylation and inactivation of transcriptionally silent regions. However, as noted above, a number of other mechanisms could also account for the difference in DNA methylation between the two groups. Future studies will be required to clarify the significance of these changes in these transcripts. Such studies would include confirmation of differences at the protein level, effects of overexpression and underexpression of the relevant proteins on DNA methylation, and studies on how the Dnmt1 mutation leads to preservation of its expression.

The observation that DNA hypomethylation occurs after 11 months of age in the control mice and correlates with the development of autoimmunity is consistent with the previously noted association between T-cell DNA hypomethylation and autoimmunity. In this case hypomethylation was caused by aging of the control mice, rather than pharmacologic treatment of T lymphocytes. This association is supported by the delayed appearance of autoimmunity and the progressive increase in DNA methylation in the knockout mice. It is also of interest that the relatively hypomethylated young knockout mice did not develop autoimmunity. This suggests either that autoimmunity develops slowly and was prevented by the compensatory methylation, or that the methylation status of genes crucial to the development of autoimmune status is tightly regulated, and is methylated normally despite a total decrease in genomic d^{m}C content.

It is possible that DNA hypomethylation contributes to autoimmunity in normal aging and our drug-induced model, because similar features are seen in both models, including the development of anti-DNA antibodies and an autoimmune liver disease with a chronic, focal inflammatory infiltrate. Renal and lung lesions were also more common in the control mice, and they are seen in the drug-induced DNA hypomethylation model (6). The features of autoimmunity in C57BL/6 mice are more pronounced when they are at 24 months of age (10), suggesting that examination of older mice might give further evidence of autoimmunity. The aged C57BL/6 mice also developed sialadenitis, which is in contrast to the drug-induced model. It is possible that the sialadenitis is a strain-specific feature, and C57BL/6 mice have not been studied in the drug-induced lupus model. Alternatively, other changes in methylation patterns associated with aging but not produced in the drug-induced model may contribute, or methylation change in the salivary glands themselves might participate.

There were also several observations indicating that the development of age-dependent changes in the immune system are different between the Dnmt1 knockout mice and controls. The memory subset, which increases with age, developed more slowly in the knockout mice. Similarly, IL-2 responses decreased more slowly with age in the knockout mice. The mechanisms causing the appearance of the memory subset and the decrease in IL-2 production with aging are unknown. A decrease in T-cell proliferative capacity in the knockout mice could explain both observations, but proliferative capacity was actually greater in the knockouts than in controls at 18 months, arguing against this interpretation. The observation that the decrease in IL-2 production and development of the memory subset is delayed in the knockout mice suggests that changes in DNA methylation may be responsible for their appearance in the control mice, and that preventing DNA hypomethylation slows their development, although other mechanisms are possible, including increased genomic instability caused by DNA hypomethylation (33). If the alterations caused by the mutation prevent or delay the development of immune senescence, an important test of this hypothesis will be to determine if mice with heterozygous Dnmt1 deficiency live longer and are more disease resistant than their wild-type littermates. These studies are in progress.

The identity of the lymphocyte genes affected by age-dependent changes in DNA methylation is of interest and highly relevant to these studies. Future studies will have to address this question. However, to approximate the number of genes affected, our group has treated human CD4+ T-lymphocyte lines with the DNA methylation inhibitor 2-deoxy-5-azacytidine, and we have compared gene expression in treated and untreated cells by using Affymetrix oligonucleotide arrays. Approximately 619 out of 7000 genes tested demonstrated a twofold or greater change in their level of expression (unpublished results). Furthermore, our group has reported that ∼15% of human T-lymphocyte genes with CpG islands are variably methylated on a clonal basis by middle age (34). These observations suggest that a relatively large number of genes could be modified by age-dependent changes in DNA methylation.

An alternative interpretation of these results is that effects of the Dnmt1 mutation on nonlymphoid tissues are responsible for the immunologic effects, preventing the development of autoimmunity and immune senescence indirectly. This seems unlikely, because adoptive transfer studies demonstrate that lymphocytes from young mice will restore immune responses in old recipients (35), and that lymphocytes from old mice will induce autoimmunity in young recipients (36). This suggests that these age-dependent phenomena are unique to the lymphocytes and are not affected by the host. Exclusion of this possibility will require adoptive transfer experiments, in which lymphocytes from the control group are transferred into the knockout mice, and retention of phenotype assessed.

In summary, in this study, wild-type control mice demonstrated an age-dependent decrease in T-cell genomic d^{m}C content and developed autoimmunity similar to that reported by our group to be induced by hypomethylated T cells, whereas the Dnmt1 mice increased lymphocyte d^{m}C content with age and demonstrated delayed development of autoimmunity. This supports the proposed association between DNA hypomethylation and autoimmunity (5,6). The observation that d^{m}C content increased with aging in the knockout mice also suggests that counteracting DNA hypomethylation may prevent immune changes with aging. Persistance of high expression of MeCP2 may also help maintain methylation levels with aging. Immune senescence is a complex area, with many potential abnormalities noted, and multiple mechanisms possible. The data presented in this report suggest that one mechanism might be changes in DNA methylation patterns, with subsequent effects on gene expression and chromatin structure.

REFERENCES

1. Bird A, Wolffe A. Methylation-induced repression—belts, braces, and chromatin. *Cell.* 1999;99:451–454.
2. Razin A, Cedar H. DNA methylation and embryogenesis. In: Jost JP, Saluz HP, eds. *DNA Methylation: Molecular Biology and Biological Significance.* Basel, Switzerland: Birkhäuser Verlag; 1993:343–357.
3. Mostoslavsky R, Bergman Y. DNA methylation: regulation of gene expression and role in the immune system. *Biochim Biophys Acta.* 1997;1333:F20–F40.
4. Turker M, Bestor T. Formation of methylation patterns in the mammalian genome. *Mutat Res.* 1997;386:119–130.
5. Quddus J, Johnson KJ, Gavalchin J, et al. Treating activated CD4+ T cells with either of two distinct DNA methyltransferase inhibitors, 5-azacytidine or procainamide, is sufficient to induce a lupus-like disease in syngeneic mice. *J Clin Invest.* 1993;92:38–53.
6. Yung RL, Quddus J, Chrisp CE, Johnson KJ, Richardson BC. Mechanisms of drug induced lupus. I. Cloned Th2 cells modified with DNA methylation inhibitors *in vitro* cause autoimmunity *in vivo. J Immunol.* 1995;154:3025–3035.
7. Golbus J, Palella TD, Richardson BC. Quantitative changes in T cell DNA methylation occur during differentiation and ageing. *Eur J Immunol.* 1990;20:1869–1872.
8. Wilson VL, Smith RA, Ma S, Cutler RG. Genomic 5-methyldeoxycytidine decreases with age. *J Biol Chem.* 1987;262:9948–9951.
9. Vanyushin BF, Nemirovsky LE, Klimenko VV, Vasiliev VK, Belozersky AN. The 5-methylcytosine in DNA of rats. *Gerontologia.* 1973;19:138–152.
10. Hayashi Y, Utsuyama M, Kurashima C, Hirokawa K. Spontaneous development of organ-specific autoimmune lesions in aged C57BL/6 mice. *Clin Exp Immunol.* 1989;78:120–126.
11. Klinman D. Similarities in B cell repertoire development between autoimmune and aging normal mice. *J Immunol.* 1992;148:1353–1358.
12. Miller RA. Immune system. In: Masoro E, ed. *Handbook of Physiology: Aging.* New York: Oxford University Press; 1995:555–590.
13. Li E, Bestor TH, Jaenisch R. Targeted mutation of the DNA methyltransferase gene results in embryonic lethality. *Cell.* 1992;69:915–926.
14. Bestor T, Laudano A, Mattaliano R, Ingram V. Cloning and sequencing of a cDNA encoding DNA methyltransferase of mouse cells. *J Mol Biol.* 1988;203:971–983.
15. Austin, H III, Muenz L, Joyce K, et al. Prognostic factors in lupus nephritis: contribution of renal histologic data. *Am J Med.* 1983;75:382–391.
16. Hobbs MV, Weigle WO, Noonan DJ, et al. Patterns of cytokine gene expression by CD4+ T cells from young and old mice. *J Immunol.* 1993;150:3602–3614.
17. Yung RL, Chang S, Hemati N, Johnson K, Richardson BC. Mechanisms of drug-induced lupus. IV. Comparison of procainamide and hydralazine with analogs *in vitro* and *in vivo. Arthritis Rheum.* 1997;40:1436–1443.
18. Deng C, Yang J, Scott J, Hanash S, Richardson B. Role of the ras-MAPK signaling pathway in the DNA methyltransferase response to DNA hypomethylation. *Biol Chem.* 1998;379:1113–1120.
19. Littell RC, Folks JL. Asymptotic optimality of Fisher's method of combining independent tests. *J Am Statist Assoc.* 1971;66:802–806.
20. Ono T, Uehara Y, Kurishita A, Tawa R, Sakurai H. Biological significance of DNA methylation in the ageing process. *Age Ageing.* 1993;22:S34–S43.
21. Ono T, Shinya K, Uehara Y, Okada S. Endogenous virus genomes become hypomethylated tissue—specifically during aging process of C57BL mice. *Mech Ageing Devel.* 1989;50:27–36.
22. Laird PW, Jackson-Grusby L, Fazeli A, et al. Suppression of intestinal neoplasia by DNA hypomethylation. *Cell.* 1995;81:197–205.
23. Ng H-H, Bird A. DNA methylation and chromatin modification. *Curr Opin Genet Dev.* 1999;9:158–163.
24. Antequera F, Bird A. Number of CpG islands and genes in human and mouse. *Genetics.* 1993;90:11995–11999.
25. Yang J, Deng C, Hemati N, Hanash SM, Richardson BC. Effect of mitogenic stimulation and DNA methylation on human T-cell DNA methyltransferase expression and activity. *J Immunol.* 1997;159:1303–1309.
26. Okano M, Xie S, Li E. Cloning and characterization of a family of novel mammalian DNA (cytosine-5) methyltransferases. *Nat Genet.* 1998;19:219–220.
27. Bhattacharya SK, Ramchandani S, Cervoni N, Szyf M. A mammalian protein with specific demethylase activity for mCpG DNA. *Nature.* 1999;397:579–583.
28. Ramchandani S, Bhattacharya SK, Cervoni N, Szyf M. DNA methylation is a reversible biological signal. *Proc Natl Acad Sci USA.* 1999;96:6107–6112.
29. Ng H-H, Zhang Y, Hendrich B, et al. MBD2 is a transcriptional repressor belonging to the MeCP1 histone deacetylase complex. *Nat Genet.* 1999;23:58–61.
30. Weiss A, Keshet I, Razin A, Cedar H. DNA demethylation in vitro: involvement of RNA. *Cell.* 1996;86:709–718.
31. Kaludov N, Wolffe A. MeCP2 driven transcriptional repression *in vitro*: selectivity for methylated DNA, action at a distance and contacts with the basal transcription machinery. *Nucl Acids Res.* 2000;28:1921–1928.
32. Yu F, Thiesen J, Strätling W. Histone deacetylase-independent transcriptional repression by methyl-CpG-binding protein 2. *Nucl Acids Res.* 2000;28:2201–2206.
33. Chen RZ, Pettersson U, Beard C, Jackson-Grusby L, Jaenisch R. DNA hypomethylation leads to elevated mutation rates. *Nature.* 1998;395:89–93.
34. Zhu-XX, Deng C, Kuick R, et al. Analysis of human peripheral blood T cells and single-cell derived T cell clones uncovers extensive clonal CpG island methylation heterogeneity throughout the genome. *Proc Natl Acad Sci USA.* 1999;96:8058–8063.
35. Orme IM. Aging and immunity to tuberculosis: increased susceptibility of old mice reflects a decreased capacity to generate mediator T lymphocytes. *J Immunol.* 1987;138:4414–4418.
36. Kanda K, Onji O, Ohta Y. Spontaneous occurrence of autoimmune cholangitis in senescent mice. *J Gastroenterol Hepatol.* 1993;8:7–14.